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Cloning and Expression of Chicken AvBD8 Mature Peptide Gene in *Lactococcus lactis*

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Abstract: Chicken Beta Defensin-8 (AvBD8) is an antibacterial peptide that plays significant roles in innate immunity. The aim of this study was to investigate the expression of Chicken avian AvBD8 mature peptide gene in *Lactococcus lactis* (*L. lactis*) system. In this study, the *AvBD8* gene was cloned and sequenced from chicken liver by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Then, the AvBD8 mature peptide gene was amplified and cloned into the expression vector pNZ8048. The result showed that the recombinant vector pNZ8048-AvBD8 that verified by colony PCR, double digestion and DNA sequencing was transformed into the competent cell *L. lactis* NZ9000 by electroporation. The expression of the recombinant protein AvBD8 was confirmed by tricine SDS-PAGE analysis. The present findings suggest that *L. lactis* could act as a host for the production of avian beta defensins for further study.

Key words: AvBD8 mature peptide, cloning, expression, *Lactococcus lactis*, China

INTRODUCTION

Antimicrobial Peptides (AMPs) are generally short cationic peptides that have potent killing activity against a range of microbes including bacteria, fungi, viruses and protozoa (Higgs *et al*., 2007). Defensins, a subset of antimicrobial peptides are cystine rich peptides that vary in length from 18 to approximately 45 amino acids and are enriched in hydrophobic and cationic amino acids residues (Zasloff, 2002). Avian Beta-Defensins (AvBDs) are now considered as one of the key components of innate immunity in avian species. A total of 14 beta-defensins genes (*AvBD1-14*) have been identified in the chicken (Lynn *et al*., 2004; Xiao *et al*., 2004). AvBD8 is one kind of the chicken beta-defensins which clustered densely within a 86 kb distance on the chromosome 3q3.5~q3.7. The encoding polypeptide with 66 amino acids consisted of signal peptide, propiece peptide and mature peptide (Ganz, 2003; Cederlund *et al*., 2011).

Several studies have been earlier reported for the biosynthesis and purification of defensins. However, the isolation of defensins from natural sources or by chemical synthesis is either inefficient or costly and time consuming. Genetic engineering has been utilized in the production of small cationic peptides for over a decade (Piers *et al*., 1993). *L. lactis* can be considered a good candidate for heterologous protein secretion because relatively few proteins are known to be secreted by *L. lactis* even in multi-deficient protease strains (Wu *et al*., 1998; Lee *et al*., 2001) and laboratory *L. lactis* strains do not produce any extracellular proteases. In the past years, impressive progress has been made in the development of genetic engineering tools and the molecular characterization of lactococci (Wood and Warner, 2003). The availability of an easy to operate and strictly controlled food grade expression system Nisin Controlled Expression System (NICE) has been developed and used for many applications (De Ruyter *et al*., 1996), then the use of *L. lactis* as bacterial systems to express heterogonous protein is becoming a promising issue. Researchers designed a study to express the recombinant AvBD8 protein in *L. lactis* using the system Nisin Controlled Expression system.

MATERIALS AND METHODS

Bacterial strains and plasmid: The *L. lactis* NZ9000 and the plasmid pNZ8048 were prepared at Institute of

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Microbiology Chinese Academy of Sciences (Beijing, China). The plasmid pGEM-T Easy was purchased from Progema (USA). The *Escherichia coli* (*E. coli*) strains DH5α was purchased from Invitrogen (Beijing, China).

Main reagents: Trizol reagent, T4 DNA ligase, Taq DNA polymerase and all restriction enzymes were purchased from TaKaRa (Dalian, China). PCR product purification kits and plasmid extraction kits were provided by Omega (Beijing, China). Protein molecular markers were purchased from Shanghai Sangon Biotech Service Co., Ltd. (Shanghai, China). M-MLV reverse transcriptase was purchased from Progema (USA).

Primer design: According to the reported cDNA gene sequence (NM_001001781) of AvBD8, two pairs of primers were designed by primer 5 program. The primers (P1: 5'-ATGAAGATCCTTTACCTTCTCTTG-3' and P2: 5'-TTAGTCGTACAGTCCGGC-3') were used for reverse transcription reaction and amplification of the *AvBD8* gene. The primers (P3: 5'-CATGCCATGGGTAACAACGA GGCACAGTGT3-3' and P4: 5'-CCCAAGCTTTTAGCTG TACACATCCGGCAG-3') were designed for two specific endonuclease enzyme site (NcoI and HindIII) based on expression vector cloning site.

RNA extraction, RT-PCR and cloning of the *AvBD8* gene: Total cellular RNA was extracted from chicken liver using Trizol reagent according to the manufacturer's instructions. The extracted RNA was resuspended in 20 μL RNase free water and stored at -70°C until use. The reverse transcription reaction mixture (20 μL) consisted of 10 μL total RNA, 4 μL RT buffer, 2 μL 10 mM of each dNTP, 40U RNase inhibitor, 2 μL oligo-dT and 50 U Rever Tra Ace. The reverse transcription was performed at 42°C for 1 h followed by heat inactivation for 5 min at 95°C. The PCR amplification was carried out in a 25 μL reaction mixture containing 0.2 μM specific primers (P1/P2), 2 μL of cDNA, 1×PCR buffer, 0.2 mM dNTP mixture and 1U Taq™. The PCR reaction was carried out as follows: an initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 57°C for 40 sec and polymerization at 72°C for 1 min. The final polymerization step was performed at 72°C for 10 min. The PCR products were analyzed by 2.0% agarose gel and then cloned into the pGEM-T Easy plasmid. The recombinant plasmid pGEM-T Easy-AvBD8 was transformed into *E. coli* DH5a competent cells.

Construction of expression plasmid: Recombinant DNA techniques were carried out according to standard methods described by Sambrook and Russell (2001). The AvBD8 mature peptide sequence was amplified by PCR using a specific complementary set of primers (P3/P4). The PCR reaction was as follows: an initial denaturation for 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 50 sec. The final polymerization step was performed at 72°C for 10 min. The purified PCR product was digested with NcoI and HindIII and ligated with the pNZ8048 that was linearized with the same enzymes. The recombined plasmids were transformed into *L. lactis* NZ9000 competent cells. In order to confirm the integrity of the insert (the AvBD8 mature peptide gene) and no errors have taken place at the ligation sites, researchers performed a direct colony PCR and extraction recombinant plasmid (pNZ8048-AvBD8) and digested it with NcoI and HindIII. Finally, the recombinant plasmid verified by double digestion was sequenced by Shanghai Sangon Biotech Service Co., Ltd.

Expression of AvBD8 mature peptide gene in *L. lactis*: A positive *L. lactis* NZ9000 recombinant transformant was cultured overnight at 30°C in 5 mL GM17 medium then the bacteria was inoculated to 500 mL GM17 medium containing 50 μg mL^{-1} chloramphenicol and then cultured at 30°C until the appropriate density (OD600 = 0.4-0.5) was reached. Nisin was added to final concentration of 1 ng μL^{-1} to induce the expression of the protein at 30°Cfor 8 h. The bacterium was centrifuged at 12000 rpm for 15 min at 4°C and then washed by 0.1 M PBS (pH7.4) for three times and the bacterium was suspended in PBS. The bacterium suspension was dealed by ultrasonic. The mixture was collected and centrifuged at 12000 rpm at 4°C for 30 min. The supernatant and the precipitation were analyzed by Tricine SDS-PAGE electrophoresis.

RESULTS AND DISCUSSION

Isolation and sequence analysis of AvBD8: Total cellular RNA was extracted from chicken liver using the Trizol reagent according to the manufacturer's instructions. Three distinct bands were found at 28, 18 and 5sec by 1% agarose gel electrophoresis (Fig. 1). The chicken *AvBD8* gene was successfully amplified by RT-PCR using designed primers (P1/P2). Analysis of PCR product on agarose gel by electrophoresis, the observed length of the target nucleotide sequence was approximately 201 bp, consistent with the expected size (Fig. 2).

Construction of the pNZ8048-AvBD8 expression plasmid: The AvBD8 mature peptide gene was amplified by PCR from the pGEM-T Easy-AvBD8 plasmid and then inserted into the expression vector pNZ8048.

Subsequently, the constructed clone was verified with a double enzymatic digestion (Fig. 3). Sequence analysis confirmed that the AvBD8 mature peptide gene sequence showed 99% homology with other AvBD8 sequences in GenBank (NM_001001781) (Fig. 4) but the deduced protein was 100% completely homologous.

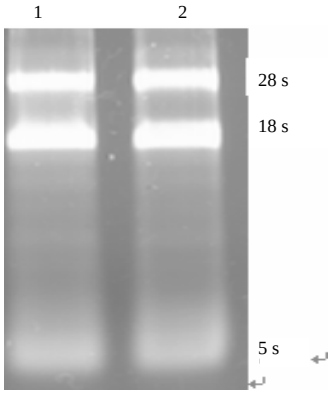

Fig. 1: Electrophoretic analysis of Sanhuang broiler. 1, 2: RNA of liver

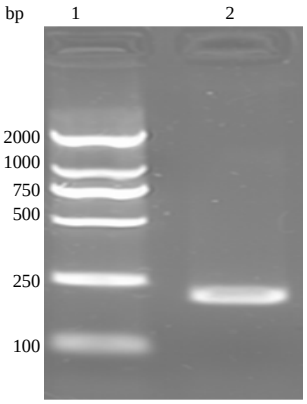

Fig. 2: Electrophoretic analysis of chicken AvBD8 RT-PCR products. 1: DL2000 DNA marker; 2: AvBD8

Protein expression in *L. lactis*: The positive colony of *L. lactis* NZ9000 which contained expression vector pNZ8048-AvBD8 was induced by 0.5 mM nisin at 30°C for 8 h. Tricine-SDS-PAGE analysis showed most of the expressed protein existed in the supernatant with soluble form and a little existed in the precipitate (Fig. 5).

Defensins act as a first line of defense against invading pathogens and execute the antimicrobial activity by non-oxidative mechanisms (Sahl *et al*., 2005). In an age when antibiotic resistance is an increasing problem, these peptides are of interest as potential novel pharmaceutical agents (Donoghue, 2003; Lynn *et al*., 2004). Chicken AvBD8 is an antibacterial peptide which has broad spectrum antimicrobial activity is expressed in epithelial cells of the liver and the gall bladder (Higgs *et al*., 2005, 2007). In this study, the gene

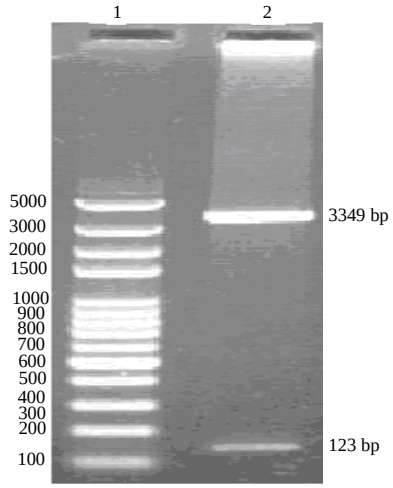

Fig. 3: Identification of recombinant plasmid by restriction enzyme digestion (NcoI and HindIII). 1: DNA marker; 2: pNZ8048-AvBD8 digestion by NcoI and HindIII

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Query 1    AACAACGAGGCACAGTGTGAGCAGGCAGGAGGGATCTGCTCCAAGGATCACTGCTTCCAC   60
           ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct 42   AACAACGAGGCACAGTGTGAGCAGGCAGGAGGGATATGCTCCAAGGATCACTGCTTCCAC   101
Query 61   CTCCATACCAGAGCCTTTGGGCACTGCCAGAGAGGGGTCCCGTGCTGCCGGACTGTGTAC   120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 102  CTCCATACCAGAGCCTTTGGGCACTGCCAGAGAGGGGTCCCGTGCTGCCGGACTGTGTAC   161
Query 121  GAC 123
           |||
Sbjct 162  GAC 164
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Fig. 4: Blast contradistinction of AvBD8 mature peptide sequence

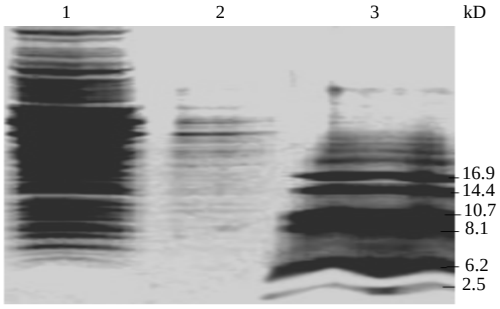

Fig. 5: AvBD8 mature peptide induced by the nisin. 1: pNZ8048-AvBD8 induction supernatant; 2: pNZ8048-AvBD8 induction precipitation; 3: Low MW standard protein marker

of chicken β-defensins-AvBD8 was cloned from chicken liver. The gene was 99% homology with the gene in GenBank (NM_001001781), however the deduced protein was 100% completely homologous.

To date, many expression systems have been developed to produce recombinant proteins for various biotechnological applications. Among prokaryotic systems, the highest protein levels are obtained using *Escherichia coli* as the cell factory (Jana and Deb, 2005). Recombinant gallinacin-9 and gallinacin-8 was successful expressed in *Escherichia coli* BL21 (DE3) strain (Ma *et al*., 2008). However, in *E. coli*, endotoxin or lipopolysaccharide should be removed from proteins to be administered to humans. The Gram positive bacterium having a generally recognized as safe status, the Lactic Acid Bacterium Model *L. lactis* is becoming an attractive alternative for heterologous protein secretion (Morello *et al*., 2008). *L. lactis* has been studied for the last 2 decades: its metabolism is relatively simple and well known (Bolotin *et al*., 2001). Plasmids have been constructed for translational and transcriptional fusions and for intracellular production or secretion of the gene product. pNZ8048 is the most commonly used plasmid for translational fusions. A gene of interest can be PCR-amplified using primers that introduce the canonical NcoI site around the ATG start codon, allowing direct cloning of the gene fused to the nisA start codon (Mierau and Kleerebezem, 2005).

CONCLUSION

In this study, the recombinant vector pNZ8048-AvBD8 was constructed and transformed into the *L. lactis* NZ9000 by electroporation. The expression protein was mainly excited in supernatant and lower in precipitation. The amino acid which is code by AvBD8 mature peptide gene is 4.5 kD but there was not the purpose of protein band. It is probably that the expression of exogenous gene in *Lactococcus lactis* is less. The optimization of expression conditions such as temperature and concentration of nisin and Western blotting which will used to detect the AvBD8 mature peptide.

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